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Nitrogen mineralization in biofuel cropping systems in soils

Yili Meng
Iowa State University

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Yili Meng

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Michael L. Thompson, Major Professor
Daniel C. Olk
Michael J. Castellano
Thomas E. Loynachan

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ABSTRACT

This thesis includes two studies. The first study compared the net N mineralization rate in soils of five cropping systems (corn-soybean rotation, continuous corn, continuous corn with a winter rye cover crop, perennial prairie, and N-fertilized perennial prairie) in Iowa. To consider the effect of plant residues and freezing-thawing on net N mineralization rate, treatments with and without plant residues and with or without freezing were established and followed through a 30-day aerobic incubation. The effect of chemical characteristics of soil samples and the plant residue samples of each cropping system on N mineralization were also investigated. Overall, the cropping systems had significant effect on net N mineralization rate: N-fertilized perennial prairie (a) ≈ continuous corn with winter rye (a) ≥ corn-soybean rotation (ab) ≥ continuous corn (bc) ≥ perennial prairie (c) (lowercase letters indicate least significant differences ($p < 0.05$). Freezing and thawing treatment increased the net N mineralization rate about twofold in the 30-day period incubation. The simple presence of plant residues did not affect net N mineralization, but the plant residue N per weight of soil was significantly correlated with N mineralization.

The second study measured the concentration of amino acids in soil and plant residues which were extracted from the same samples as the first study. Amino acids were analyzed by using two approaches: high performance liquid chromatography (HPLC) which couples cation exchange chromatography with a ninhydrin colorimetric method, and high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). One objective of this study was to compare the precision and effectiveness of the two approaches. Another objective of this study was to

document the concentration of amino acids in partially decomposed plant residues. The concentrations of amino acids in all the soil samples were similar, as measured by the amperometric method and the ninhydrin method. On the other hand, in plant samples, the ninhydrin method could be a better choice than the amperometric method at present, because of the peaks of some amino acids were overlapped by co-eluting carbohydrate peaks. About forty percent of total N in the partially decomposed plant residues was amino acid-N. Compared to the freshly dried plant samples, the total amino acid concentration of the dried and partially decomposed plant residues (mainly roots) was lower.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Background

1.1.1 Importance of nitrogen

Nitrogen (N) is an essential nutrient for plant growth because it is an important component of chlorophyll, proteins, adenosine triphosphate (ATP), and nucleic acids. Chlorophyll is involved in photosynthesis; proteins serve as enzymes or are integral to structural units in plant cells; ATP is the compound involved in intracellular energy transfer; and nucleic acids are utilized in transmitting and expressing genetic information.

Nitrogen is often the most limiting nutrient for crop production. Yield reductions have been reported due to N deficiency in agricultural soils. However, excess applied fertilizer-N causes lower N use efficiency. The N fertilizer that is not used by the crop may leach through soils, causing contamination of groundwater or surface water (Almasri and Kaluarachchi, 2004), and N also may be emitted from soil as a greenhouse gas. Therefore, study of soil N dynamics is necessary to optimize N fertilizer recommendations for agronomic, economic, and environmental reasons.

1.1.2 Nitrogen dynamics in soil

Input of N to soil can occur through biological fixation, by fixation of atmospheric N_2 by lightning, and by N fertilizer application. Biological nitrogen fixation includes symbiotic fixation by microorganisms in association with legumes, symbiotic fixation with non-legumes, and non-symbiotic fixation. Symbiotic fixation of N with

legumes can contribute considerable N to crop production; in the U.S., the amount of biological N_2 fixation is approximately 1/3 of the amount of fertilizer N applied (Havlin et al. 1999). Besides fixation and fertilizer application, inorganic NH_4^+ and NO_3^- dissolved in rain water also contribute a small amount of N to soils.

Output of N from soil loss occurs through crops harvest removal, leaching, ammonia volatilization, and gas emission by the denitrification process. N loss through leaching is primarily in the form of nitrate (NO_3^-). The mobility of nitrate makes it vulnerable to leaching that can cause serious ground water contamination. Ammonia volatilization increases with soil pH. Denitrification is an anaerobic respiration process that reduces NO_3^- to gaseous N products; it increases with poor drainage and high temperature. The gases emitted by denitrification include NO_x, N_2O, and N_2. NO_x [NO and NO_2 (nitric oxide and nitrogen dioxide)] in the atmosphere may be oxidized and dissolved in rain water and returned to the soil. N_2O is a greenhouse gas with significant global warming potential.

Other N transformations in the soil include N mineralization (ammonification), nitrification, and immobilization. N ammonification refers to the conversion of soil organic N compounds such as proteins, amino sugars, and nucleic acids to ammonium (Paul and Clark, 1989). It is mediated by enzymes produced by microbes and soil animals. Nitrification is the oxidation process in which certain groups of microbes convert NH_4^+ to NO_2^- and eventually to NO_3^-. Nitrifying organisms are mainly chemoautotrophic bacteria. The energy produced in the oxidation process promotes growth of the nitrifying organisms. N immobilization is the opposite of nitrogen mineralization; it may also be called N assimilation. Soil microbes assimilate inorganic

N, converting it to organic N. The balance of concurrent N mineralization and N immobilization is called net N mineralization. Ammonium-N and nitrate-N produced by net N mineralization are important sources of N for crop uptake.

1.1.3 Importance of N mineralization and measurements of mineralization

This thesis is focused mainly on N mineralization in soil. The N required by non-leguminous crop growth is supplied both by N fertilizer and by N mineralized from crop residues and soil organic matter. Therefore, it is necessary to accurately estimate the amount of N produced by mineralization. In the laboratory, N mineralization can be measured by incubating soil at a constant temperature either aerobically or anaerobically. The soil is leached with a dilute salt solution after incubation, and the increase in inorganic N in the leachates compared to zero time incubation represents the net amount of N mineralized, i.e., gross mineralization minus assimilation or immobilization.

1.2 Thesis organization

This thesis follows the journal paper format. Chapter 1 includes the general introduction to the thesis. Chapter 2 is a manuscript that will be submitted to a soil science journal. Chapter 3 is an additional study about methods of determination of amino acids in soils and plant materials. Chapter 4 comprises general conclusions from the journal paper and the amino acid study. The final two sections entail acknowledgments and an appendix.

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CHAPTER 2. THE EFFECT OF CROPPING SYSTEMS ON NET NITROGEN MINERALIZATION

A paper prepared for submission to a soil science journal

Yili Meng, Teresita Chua-Ona, and Michael L. Thompson

2.1 Abstract

Plant-available N in soil originates both from commercial fertilizer N and from organic N mineralization. The mineralizable organic N comes from soil organic matter and crop residues. Better predictions of N mineralization rates will be essential to make better management decisions for cropping systems. This study compared the net N mineralization rate in five cropping systems (corn-soybean rotation, continuous corn, continuous corn with a winter rye cover crop, perennial prairie, and N-fertilized perennial prairie) in Iowa. To consider the impact of plant residues on mineralization, soil net N mineralization rate was measured with and without plant residues. In addition, some soil samples were frozen and thawed before measuring the N mineralization to explore the effect of freezing and thawing in the field on N mineralization. Net N mineralization was measured by a method similar to that of Stanford and Smith (1972). Thirty g (oven-dry based) of field moist soil were place in a column and incubated under aerobic conditions at 35 ℃ for 30 days. The soil column was leached every five days with 5 mM $CaCl_2$ solution, followed by equilibration with a nutrient solution that contained no N. The cumulative inorganic N in the leachate was calculated to determine the net mineralization rate. Cropping systems had a significant effect on net N mineralization rate, in the

following order: N-fertilized perennial prairie (a) ≈ continuous corn with winter rye (a) ≥ corn-soybean rotation (ab) ≥ continuous corn (bc) ≥ perennial prairie (c) (lowercase letters were assigned based on least significant differences ($p < 0.05$)). The freezing and thawing treatment increased the net N mineralization rate about twofold during the 30-day incubation period. The presence of plant residues did not affect net N mineralization, but N mineralization was significantly and positively correlated with the plant residue N per weight of soil.

2.2 Introduction

Nitrogen fertilizer is usually a major cost of managing cropland, but not all the added N is taken up by plants. The use efficiency of N fertilizer in agricultural crops is seldom more than 50% (Singh and Ryan, 2015). Nitrogen not taken up by plants may contribute to nitrate leaching through soils, causing serious contamination of groundwater and surface water (Almasri and Kaluarachchi, 2004; Puckett et al., 2011; Williams et al., 2015). Excess applied fertilizer-N can also be lost to the atmosphere through denitrification or ammonium volatilization. Nitrate (NO_3^-) in soil can be reduced to nitrite (NO_2^-) by denitrifiers, and nitrite can be further reduced to nitric oxide (NO) gas, nitrous oxide (N_2O) (a strong greenhouse gas), and N_2 gas. Ammonium (NH_4^+) cations can be adsorbed to soil clay particles and soil organic matter, achieving equilibrium with NH_4^+ in the soil solution. But with increasing soil pH and temperature, ammonia volatilization increases, especially in soils of low CEC. Soil NH_4^+ can also be transformed to NO_3^- through nitrification, and then nitrate can be lost due to denitrification. However, under some management systems, fertilizer N is incorporated into organic matter. In that form,

it may be slowly mineralized to a plant-available form, and there may be less loss of N by leaching. To reduce the application rate of N fertilizers and to mitigate the environmental concern of nitrate leaching, accurate estimates of N availability under various cropping systems are necessary to make better management decisions.

Plant-available N in soil originates both from commercial fertilizer N and from organic N mineralization. The mineralizable organic N may come from soil organic matter, crop residues, and other organic wastes. Numerous studies have been conducted to investigate the factors that affect N mineralization rates in soils. In this study, we were primarily interested in the effects of cropping systems on N mineralization.

Cropping systems can affect N mineralization by changing soil chemical and biological properties. Cropping systems that include perennial species are expected to add C and N to the organic N pool of surface soil horizons because perennial systems build considerable belowground biomass (Davis et al., 2010; Davis et al., 2013). More belowground biomass produced by perennial species increases the potential for N mineralization by enriching the biologically active N pools in soils (Deng and Tabatabai, 2000; Carpenter-Boggs et al., 2000). Nitrogen mineralization potential is related to the amount of crop residues returned to the soil (Christenson and Butt, 1997; Laird and Chang, 2013). Christenson and Butt (1997) measured N mineralization potentials (N_0) under four cropping systems (4 combinations of crop rotations included corn, sugarbeet, navy bean, and oat) in a Misteguay silty clay in Michigan. They reported that variations among the cropping systems in the amount of residues returned to the soil led directly to variations in N mineralization potentials. Using an exponential model, they also reported that the N mineralization potential increased 1 mg kg^{-1} for each 0.33 Mg ha^{-1} of crop

residue in the soil. Laird and Change (2013) investigated N mineralization potentials (N_0) of soils in fields cropped to corn and soybean in Minnesota. In the upper 15 cm of the soil, they found that removal of all crop residues led to reductions in soil organic C, total N, N mineralization potential, and cation exchange capacity.

A winter cover crop of cereal rye may be used to scavenge residual soil inorganic N after the growing season, so that N may be released at an early growth stage of the subsequent crop. Therefore cropping systems that include winter cover crops are expected to have higher N mineralization rates than those systems without winter cover crops (Ranells and Wagger, 1996; Hu et al., 1997; Dabney et al., 2010).

Besides the amount of crop residues, the chemical composition of the residues may also exert an important influence in N mineralization in soils. The N concentration and C/N ratio of crop residues are determinants of the N release, and they are often used to predict the net effects of crop residues on soil mineral N dynamics (Trinsoutrot et al., 2000; Seneviratne, 2000). Net N immobilization often dominates in plant residues with N concentration up to about 1.7—1.8% N (Constantinides and Fownes, 1994). It has also been reported by many authors that net N mineralization occurs when C/N ratios of residues are < 25 (e.g., Trinsoutrot et al., 2000; Kumar and Goh, 2003).

The experiments described in this thesis are part of the Comparison of Biofuel Cropping Systems (COBS) project at Iowa State University. The COBS project provides long-term, side-by-side comparison of corn- and perennial-based cropping systems. At the COBS site, it is possible to quantitatively investigate the effect of long-term cropping systems on N mineralization at the same site and under the same meteorological

conditions. Quantitative assessments of N mineralization can be used to improve mathematical models of N dynamics for each cropping system.

The effect of plant residues was also one of the factors we intended to investigate. The decomposition processes of many crops' residues have been documented by numerous studies. In most of these studies, a measured amount of fresh or dried crop residues was mixed with soil and then incubated under laboratory conditions (e.g., Constantinides and Fownes, 1994; Kumar and Goh, 2003; Hadas et al., 2004; Nakhone and Tabatabai, 2008). However, amending soil samples with crop residues in this way does not necessarily reproduce the real interactions between soils and crop residues under field conditions. In the Midwestern United States, it is common to leave crop residues in the field after harvest. When the subsequent growing season begins, the residues are already partly decomposed. In this experiment, we looked at the effect of the existing, partly decomposed crop residues on N mineralization. Net N mineralization was measured in soil samples as collected (with partly decomposed residues) and in soil samples from which visible crop residues were removed. In addition, the chemical characteristics of the partly decomposed crop residues were measured. Thus we hoped to more realistically simulate N dynamics in the soil in the early part of the crop growing season.

Soil characteristics are also likely to affect soil N availability to crops. N mineralization rates have strong positive relationships with soil organic C, soil N concentrations, and soil C/N ratio (Ros et al., 2011; McDonald et al., 2013). Some chemical methods are used to extract an N fraction from soil that can be related statistically to mineralizable N, such as extraction with hot KCl (Gianello and Bremner,

1986) or the Illinois soil N test (ISNT) (Williams et al., 2007; McDonald et al., 2013). These chemical methods are cheaper and quicker than biological incubation methods (Stanford and Smith, 1972).

Soils in mid-latitude climates such as that of Iowa may be subjected to freeze-thaw cycles in late winter and early spring. Freezing of soils could decrease microbial biomass (Larsen et al., 2002), causing turnover of soil organic matter and thus affect N mineralization (Matzner and Borken, 2008). It has been reported that net N mineralization rates increased shortly after freezing and thawing cycles (Deluca et al., 1992; Herrmann and Witter, 2002). To improve our understanding of N mineralization dynamics in spring, we also investigate the effect of freezing in this study.

Objectives

The first objective of this study was to determine and compare net N mineralization rates in five Midwest cropping systems. The second objective was to evaluate the effects of the quantity and the chemical characteristics of the decomposing plant residues of each cropping system on N mineralization. The third objective was to determine whether soil characteristics affected N mineralization. The fourth objective was to evaluate the freezing-thawing effect on N mineralization.

Definitions of terms

Gross N mineralization is usually called ammonification. This term refers to the conversion of organic N compounds to ammonium. The process is mediated by enzymes produced by microbes and soil animals.

N immobilization is the opposite of gross N mineralization and is the assimilation of ammonium by microbes.

Net N mineralization is the balance of concurrent ammonium production and consumption. Net N mineralization can be measured as the increase (or decrease) in inorganic N, including ammonium and nitrate. Measurements often assume that ammonium can be transformed to nitrate (perhaps in ~5 days) by nitrifiers in the soil. The analysis is often limited to nitrate-N only, as the other forms of N (ammonium-N and nitrite-N) are either negligible or not detectable (Deng and Tabatabai, 2000).

Potentially mineralizable N. Potentially mineralizable N is the fraction of organic N that can be converted to inorganic forms. It represents the fraction of N that is easily decomposable by soil microorganisms and is considered an indirect measure of N availability in the soil. Mineralizable N could be derived from both crop residues and from soil organic matter.

Partly decomposed plant residues. Crop residues, including stems, leaves, and roots, are commonly left in the field after harvest. Soil samples of this experiment were collected in early June of 2013, so the crop residues investigated had been left in the field for a winter and spring and were partly decomposed.

2.3 Methods and materials

2.3.1 Site description

The Comparison of Biofuel Systems (COBS) site is located in Boone County, IA, USA (41° 55′ N, 93° 45′ W). The 9-ha experimental site was in a corn-soybean rotation prior to the establishment in 2007. The site is comprised of 24 plots (61 m×27

m each), and the experimental design includes five cropping systems with four replications of each treatment, arranged as a randomized complete block design. The five cropping systems, with treatment abbreviations shown in parentheses, are (1) continuous corn grown for both grain and stover (CC); (2) continuous corn grown for grain and stover with rye (*Secale cereale* L.) as a winter cover crop (CCW); (3) corn-soybean annual rotation (CS); (4) reconstructed, multispecies prairie grown for aboveground biomass (P); and (5) N-fertilized reconstructed, multispecies prairie grown for aboveground biomass (PF). In 2012 (the year before our sampling), each CC plot received a total (pre-plant plus side-dress) N application of 199 kg ha^{-1}, each P plot received a total N application of 84 kg ha^{-1}, while each CCW plot received a total N application of 221 kg N ha^{-1} as liquid UAN (32% N). In 2012, the crop grown in the sampled CS plots was soybean (N fertilizer was not applied in 2012), so the residues collected with the soil samples included soybean residues as well as any residual corn residues from previous years. The pre-plant N fertilizer application rate was estimated from the harvest removal N. The side-dress N fertilizer application rate was estimated by the soil late spring nitrate test (Blackmer et al., 1989).

The predominant soils at the site are Webster silty clay loam (fine-loamy, mixed, superactive, mesic Typic Endoaquoll) and Nicollet loam (fine-loamy, mixed, superactive, mesic Aquic Hapludoll) distributed on a generally flat slope (mostly < 1%, with 2-3% in some small areas) (Andrews and Dideriksen, 1981).

2.3.2 Soil collection

Soil samples were collected in June 2013, after the crop was planted, but before applying a side-dressing of N fertilizer. The crops were about 15 cm tall when the samples were collected. Twenty cores per plot were collected from the 0-15 cm depth by using a 1.7-cm inner diameter steel step probe. The cores were combined in an 18-L plastic bucket and mixed by hand before subsamples were withdrawn and stored in a 4-L re-sealable plastic bag. The bags were kept cool until ready for transport to the laboratory.

2.3.3 Preparation of soil samples and plant residues

With plant residues (WR) treatment and without plant residues (WOR) treatment

Upon arrival in the laboratory, the field-moist soil samples were passed through a 4-mm sieve, and materials larger than 4 mm were removed. Each sample was split into two bags - one bag was designated as a treatment with residue (WR) (i.e., the soil material as sampled), and the other bag was designated as a treatment without residue (WOR). For the WOR sample, fresh and partly decomposed roots as well as humified plant materials were hand-picked from the soil. Excess soil material was brushed off the residues, and disposed. Then the residues were placed in a separate plastic bag. A subsample of each soil sample was air-dried and ground to pass a 2-mm sieve for chemical characterization. Moisture contents were determined in both WR and WOR soil samples.

Plant residues that were separated from the WOR treatments were washed with distilled water to remove adhering soil materials before they were oven-dried at 70 ℃ for

3 d. They were then cut into about 1-cm lengths for coarse grinding and then re-ground to < 0.1 mm using a stainless steel ball mill. Air-dried soil samples (< 2 mm), on the other hand, were further ground using 60-mL thick-walled glass jars equipped with steel rods that were allowed to gently roll on a conveyor belt for 8 h and then passed through a 0.1 mm-sieve.

Freezing treatments

The mineralization study was conducted in two batches, one after the other. Soil samples with and without residues (WR and WOR) were stored in the cold room (at 4 °C) for about 1 month before setting up the first batch of the mineralization study. The first run of the mineralization study used soil samples from three field replicates of all cropping systems in the WOR treatment and one field replicate of all cropping systems in the WR treatment. After the first run of the mineralization-incubation study, WR and WOR soil samples were frozen (at - 4 °C) for about 2 weeks before the second run of the mineralization study. The second run of the mineralization study used soil samples from three field replicates of all cropping systems in the WR treatment and one field replicate of all cropping systems in the WOR treatment. Therefore the first batch was not frozen before incubation, and the second batch was frozen before incubation. In all cases, the frozen samples were thawed at room temperature for 1 day before being used in the incubation experiments that are detailed below.

2.3.4 Soil characterization

Particle size analysis

By using the procedure of Kettler et al. (2001), the particle size distribution of soil samples was determined without pre-treatment to remove organic matter. Thirty g of sample (< 2 mm) were equilibrated with 90.0 mL of 3% (by mass) sodium hexametaphosphate solution for 2 h using a reciprocating shaker. The soil solution was passed through a 53-μm sieve supported by a wide-diameter plastic funnel, and the filtrate was collected in a large pre-weighed glass beaker. The portion that remained on the sieve (the sand fraction) was transferred quantitatively to a 50-mL, pre-weighed glass beaker. The oven-dry weight of the sand fraction was determined after drying at 110°C. The suspension containing the silt and clay was shaken well and allowed to settle for >90 min. Material that remained in suspension was less than about 4 μm in diameter (according to Stoke's Law) and was assumed to represent the clay fraction of the soil sample. The suspended clay was decanted and discarded. The silt fraction (4 – 53 μm) was transferred to another pre-weighed beaker, dried at 110 ℃, and weighed to constant weight. The clay concentration was determined by subtracting the weight of the sand plus silt from the initial weight of the soil analyzed.

Exchangeable N

A field-moist soil sample (< 4 mm), equivalent to 10 g of oven-dried sample was weighed into a 250-mL polypropylene centrifuge bottle and equilibrated with 100.0 mL of 2 *M* KCl for 1 h. The suspension was centrifuged, and the supernatant was passed through a Whatman No. 5 filter paper which was pre-washed with deionized water

(Keeney and Nelson, 1982). The filtrates were kept frozen until they were analyzed for NH_4^+ and NO_3^-, as described below.

Total C and N of soil and plant residues samples

Fifteen mg of soil (< 0.1 mm) or 2 mg of finely ground plant residue (< 0.1 mm) were weighed with an equivalent amount of tungsten oxide into a Sn capsule. Soil or plant residue samples was analyzed for total C and N by dry combustion using an automated vario MICRO cube CN analyzer (Elementar, UK). Two mg of acetanilide was run as standard at the same time the samples were run. Soil total C and N was measured both in samples with plant residues (WR) and also in samples without plant residues (WOR). All measurements were conducted in duplicate.

Soil total C was assumed to represent organic C. Because samples from all the plots had similar pH values, ranging from 6.5 to 6.9, inorganic C was assumed to be negligible. Soil organic N was calculated by subtracting the sum of ammonium-N and nitrate-N from the total soil N. The calculation was performed separately for the with-residue samples (WR) and the without-residue samples (WOR).

2.3.5 N mineralization study

Column set up

Nitrogen mineralization was investigated using a modified Stanford and Smith (1972) procedure (Chae and Tabatabai, 1986). Soil was packed in an open-top glass tube (3.5-cm dia and 15 cm long). The bottom of the glass tube was fitted with an open, narrow, glass stem that was 0.6 cm diameter and 5 cm long. A field-moist soil sample (<

4 mm) equivalent to 30 g (oven-dry basis) sample was mixed with 30 g of acid-washed, 0.84-mm sand, and 15 g of acid-washed glass beads. The soil-sand-glass bead mixture was packed into the glass tube on top of a 1-cm thick glass wool bottom support. The soil columns had an average height of 10 cm. A glass wool fiber plug (about 1 cm in thickness) was placed over the soil column to inhibit soil dispersion when leaching solution was added. A glass wool plug (about 2 cm long) was also inserted into the tip of the 5-cm long glass stem to prevent any loss of soil materials from the leaching tip. Before incubation at 35 ℃, the tubes were covered with Parafilm bored with a 5-mm diameter hole.

At the end of each equilibration period, the soil columns were leached with 100 mL of 5 mM $CaCl_2$ in 5 increments. When the last $CaCl_2$ increment was poured, the glass tube was placed on a 250-mL suction flask, and a suction of 80 kPa was applied to leach the remaining $CaCl_2$. Each leaching event was followed by adding 20 mL of a nutrient solution that contained no N. This nutrient solution was made up of 5 mM $MgSO_4$ and 5 mM KH_2PO_4, adjusted to pH 7.2 with KOH or H_2SO_4 (Carpenter-Boggs et al., 2000), to provide the nutrients other than N for soil microbial growth. Then a suction of 80 kPa was applied again to remove excess nutrient solution and to equalize the soil water potential of all samples before incubation. The gravitational water content after 80 kPa suction was about 25%. The moisture contents of the columns were adjusted by weighing the columns every 2 d and by adding deionized water to maintain a constant weight.

The leaching procedure was repeated approximately every 5 days for 30 days. The incubation-leaching procedure was extended up to 90 days for selected samples. The

incubation was terminated by leaching the soil column with 100 mL of 2 M KCl instead of the 5 mM $CaCl_2$.

The leachate collected was made up to 100 mL with deionized water before it was filtered through a 0.45-µm membrane filter paper. The filtered leachates were frozen for later analysis for NO_3^- and NH_4^+. After the initial leaching event, NH_4^+ levels in the later leachates were negligible or below the detection limit, so only nitrate was analyzed on the subsequent leachates using a microplate reader method as described below.

Nitrate analysis of leachate

Fifty µL of leachate were pipetted in duplicate into a well (on a plate having 12 x 8 microplate wells), and 250 uL of VCl_3 were added to each well as color reagent. This color reagent was prepared by dissolving 2.5 g of vanadium trichloride, 1 g of sulfanilamide, and 0.05 g of N-(1-naphthyl) ethylenediamine dihydrochloride in 1 L of 0.5 *M* HCl. The plates were placed on a platform shaker to mix the contents, and then they were kept in the dark for 14 - 16 hours for optimal color development. The plates were placed on the shaker again to mix contents before being read on a microplate reader equipped with a 540-nm filter (Hood-Nowotny et al., 2010). Nitrate standards ranging from 0 to 5 mg/L N (prepared with KNO_3 in a matrix of 5 mM $CaCl_2$) were analyzed together with the samples. Samples that exceeded the absorbance reading of 5 mg/L of N standard were diluted in 5 mM $CaCl_2$ before a 50-µL aliquot was taken for analysis. Each leachate was measured for nitrate in duplicate, and the duplicate measurements were averaged for statistical analysis.

Organic nitrogen in the leachates

Total N was determined on one group of the leachates by digesting the leachates with potassium persulfate ($K_2S_2O_8$), and then nitrate in the digests was analyzed by the microplate reader method. The difference of nitrate-N between the potassium persulfate digested leachate samples and the undigested leachate samples was calculated to represent organic N in the leachates.

We found the organic N in the leachates did not vary consistently among the cropping systems, and there was no clear trend in the fraction of total N composed of organic. Because of the significant variability, we did not determine the total N in all the leachates.

2.3.6 Calculation and statistical analyses

Nitrate-N values were summed after each leaching to express the cumulative mineralized N. The nitrate-N obtained in the initial leachates was not included in the cumulative mineralized N. We assumed that the initial leaching process removed all exchangeable-N in the soil. (The regression of the nitrate-N obtained in 5 mM $CaCl_2$ initial leaching *vs.* in 2 M KCl extraction before incubation is presented in Fig 1 of the appendix). Net N mineralization rate is the slope of the zero-order kinetics relationship of the cumulative mineralized N *vs.* incubation time. The fraction of soil organic N mineralized is the calculated by dividing the 30-day cumulative mineralized N by soil organic N.

Analysis of main effects and interactions was completed by using generalized linear mixed models (GLIMMIX) in SAS 9.4 (SAS Institute Inc., Cary, NC).

Significance of the treatments was determined by using analyses of variance and least significant difference analysis. In the statistical analysis, block effects were considered when comparing the treatments.

2.4 Results and discussion

2.4.1 Soil properties

The characteristics of soil samples collected from the cropping systems plots are given in Table 1. Soil organic C and total N in soil were not different among cropping systems. Extractable NH_4^+ was low ($\leq$ 1 mg N kg^{-1}) in all cropping systems. Extractable NO_3^- in the two reconstructed prairies cropping systems (0.7 mg N kg^{-1} in P treatment and 11.8 mg N kg^{-1} in the PF treatment) was significantly lower than in the CC (28.7 mg N kg^{-1}) and CCW (30.8 mg N kg^{-1}) cropping systems.

2.4.2 Nitrogen mineralization potential: First-order kinetics *vs.* zero-order kinetics

Potentially mineralizable N refers to the amount of N that can be mineralized under optimum and constant environmental conditions. Long-term aerobic incubation in the laboratory provides a standardized method of assessing the potential long-term N supplying capacities of soils (Stanford and Smith, 1972). Most laboratory experiments concerning N mineralization are incubated at 35 ^oC and at about 80% of field water holding capacity, considered the ideal temperature and matric potential for maximum N mineralization (e.g. Deng and Tabatabai, 2000; Carpenter-Boggs et al., 2000). N mineralized and measured in the laboratory represents potential N mineralization.

In experiments where the incubation period lasts longer than 20 weeks, the rate of soil organic N mineralized over time may start to decrease, and the cumulative mineralized N may begin to level off. In these instances, Stanford and Smith's (1972) first-order kinetic model is often derived to solve for N mineralization potential. The first-order kinetic model, which has the equation $N_m = N_o [1 - e^{-kt}]$, relates cumulative N_m (cumulative mineralized N) to t (time) to solve for N_o (N mineralization potential) and for k (a first-order rate constant) (Stanford and Smith, 1972; Stanford et al., 1974).

However, in this study, the net *N* mineralization rate did not decrease during the incubation period, perhaps because the incubation period was not long enough to reach the point that the net N mineralization rate started to decrease. Another reason could be that the most readily mineralizable N had already been mineralized by the time of soil sampling, either during the previous fall or the early spring. Therefore, an approach of zero-order kinetics model is used to solve the N mineralization rate potential other than Stanford and Smith's (1972) first-order kinetics model.

The cumulative (30-day) mineralized N was linearly related to the incubation time, with R^2 values about 0.9 (Fig. 1). Although the data are not shown in Fig. 1, we found that the cumulative mineralized N was also linearly related to time over an extended incubation period of 90 days. As shown in Fig. 1, the slopes of the lines of cumulative mineralized N *vs.* time were calculated as net N mineralization rate. (One exception to the linear rates occurred in one of the replicates of the P treatment in which N mineralization was slightly delayed at the beginning of the incubation period.) The net N mineralization rate calculated through the zero-order kinetics model was used to characterize and compare the potential N mineralization rates of the five cropping

systems over the ~30-day period. Subsequent discussions will focus on the net N mineralization rate, and the fraction of soil organic N mineralized in 30 days.

2.4.3 Net nitrogen mineralization rate

The three sample treatments were the five cropping systems, whether the soil samples were frozen or not frozen before incubation, and whether the samples were incubated with (WR) or without (WOR) plant residues. The analysis of variance of all the observations explored the effects of the three treatments on net N mineralization rate (Table 2). The cropping systems ($p = 0.0038$) and the freezing treatment ($p < 0.0001$) had significant effects on net N mineralization rate, and the presence of plant residue treatment (WOR and WR) did not significantly affect net N mineralization rate.

Effects of the presence of plant residues and freezing

The impact of plant residues on the net N mineralization rate was investigated separately for samples that had been frozen before incubation and for samples that were not frozen (Fig. 2). When data from the paired WOR and WR treatments were compared in the no-freezing treatment, the net N mineralization rate was not different (*t*-test, $n = 20$, $p = 0.38$) between the WOR (mean of 0.84 mg N kg^{-1} day^{-1}**)** and WR (mean of 0.82 mg N kg^{-1} day^{-1}) treatments. For the frozen samples, the net N mineralization rate was also not significantly different (*t*-test, $n = 20$, $p = 0.83$) between the WOR (mean of 1.6 mg N kg^{-1} day^{-1}**)** and the WR (mean of 1.6 mg N kg^{-1} day^{-1}) treatments. On the other hand, when the plant residue treatments were lumped together, the samples that were frozen before incubation had a net N mineralization rate about two times larger than that of the samples that were not frozen (*t*-test, $p < 0.00001$).

The freezing treatment resulted in a significant increase in the N mineralization rate. Deluca et al. (1992) also found that, when soil samples from Iowa were frozen and then thawed before incubation at 25^{o}C, the N mineralization increased up to twofold and remained at a higher rate up to 20 days. Herrmann and Witter (2002) studied soil samples from Sweden in modeling the freeze-thaw cycles in late winter and early spring before laboratory incubation, and they also found that freezing and thawing increased N mineralization two to three fold in a 40-day incubation. Our results are consistent with those reported by Deluca et al. (1992) and Herrmann and Witter (2002).

Freezing and thawing of soil can cause biological and physical perturbations of the soil. These include release of soluble organic materials, and the rupture of microbial cells (Morely et al., 1983). The organic material released could be easily decomposable after a freezing-thawing cycle. Ivarson and Sowden (1966) showed that the concentration of free amino acids in soils increased as a result of freezing to – 14 ^{o}C, but the source of the increased free amino acids was not well understood. The freezing treatment also decreases soil water potential; soil organic matter that is associated with clay minerals may be dehydrated and dissociate from the clay minerals while freezing. This process would increase the surface area of soil organic matter, perhaps making it more easily decomposable.

The decomposition of killed microbial cells by freezing may be a source of the increase in N mineralization rate (Schimel and Clein, 1996; Herrmann and Witter, 2002). The microbial community that survives freezing may quickly recover after thawing and feed on the dead microbial cells, releasing inorganic N. The soil microbial biomass N is about 4% of total soil N, ranging from about 2 to 6% (Brookes et al., 1985). The average

percentage of N mineralized was about 1.2% of total soil N (12 g mineralized N kg^{-1} soil N) under no-freezing treatment, while the average value percentage under freezing treatment was about 2.6% (26 g mineralized N kg^{-1} soil N) (Table 4). The average percentage of mineralized N increased by about 1.4% due to freezing, and the increase does not exceed the likely amount of microbial biomass N. We suggest that the killed microbial cells could have been an important source of organic N that was available for mineralization after the freezing-thawing cycle. However, it is thought that the increase in mineralization is a short-term phenomenon, and the amount of mineralized N after thawing is usually small in relation to the expected annual mineralization rate (Herrmann and Witter, 2002; Matzner and Borken, 2008).

Effect of cropping systems

The impact of cropping systems on the N mineralization rate was investigated separately for samples that had been frozen before incubation and for samples that were not frozen (Fig. 3). In the unfrozen samples, the net N mineralization rate varied significantly among cropping systems, ranging from 0.68 mg N kg^{-1} day^{-1} in the prairie (P) systems to 1.03 mg N kg^{-1} day^{-1} in the fertilized prairie (PF) systems. In the unfrozen samples, the net N mineralization rate in the fertilized prairie (PF) and the continuous corn with winter rye (CCW) systems were significantly larger than the rate in the continuous corn (CC) and prairie (P) systems. After the freezing treatment, the average net N mineralization rate also varied among the cropping systems, ranging from 1.3 mg N kg^{-1} day^{-1} in the continuous corn (CC) systems to 1.9 mg N kg^{-1} day^{-1} in the fertilized prairie (PF) systems (Fig. 3). After the freezing treatment, the net N mineralization rate in the fertilized prairie (PF) was significantly larger than the rate in the continuous corn

(CC) system. Overall, the variation among cropping systems in the freezing treatment was less differentiated than the variation among cropping systems in the no-freezing treatment. Among the cropping systems, freezing samples before incubation increased the net N mineralization rate the most in the prairie (P) system.

Over time, cropping systems based on perennial grass build considerable mineralizable belowground biomass, and so they are expected to have greater N mineralization rates than corn-based systems (Davis et al., 2013). Overall, we found that soil from the N-fertilized prairie cropping system (PF) released N at a faster rate than did soil from the continuous corn cropping system (CC) under both freezing and no-freezing treatments. But the N mineralization rate of the prairie system that received no N fertilizer (P) was significantly smaller than the mineralization rates of three fertilized cropping systems (PF, CS, and CCW), and somewhat smaller than the rate of the CC cropping system, when the soil was not frozen before incubation,

Some studies have reported that inclusion of a legume in a crop rotation increased net N mineralized (Carpenter-Boggs et al., 2000; Deng and Tabatabai, 2000). However, in the present study, there was not a significant difference between the CS (corn-soybean rotation) system and the CC (continuous corn) system.

Our observations matched the expectation that row-crop systems that include a winter cover crop would have a higher N mineralization rate than cropping systems without a winter cover crop (Ranells and Wagger, 1996; Hu et al., 1997). Under the no-freezing treatment, the N mineralization rate of the cropping system of continuous corn with winter cover crop (CCW) was significantly larger than the rate of the continuous corn (CC) cropping system. The N stored in winter cover rye residue may be released by

mineralization in the spring, significantly increasing the net N mineralization rate compared to row-crop systems with no cover crop.

2.4.4 Effect of soil characteristics on N mineralization

To isolate the impact of N mineralization from soil organic matter, we explored the effects of soil characteristics on N mineralization in samples without plant residues (WOR) and under the no-freezing treatment (Table 3). The net N mineralization rate was weakly related to soil C/N ratio ($p = 0.1$, $r = -0.41$), weakly related to soil organic C ($p = 0.07$, $r = -0.35$), moderately related to soil total N ($p = 0.04$, $r = -0.30$), and strongly related to clay content ($p = 0.005$, $r = -0.54$). In contrast, there was no statistical relationship ($p > 0.1$) between the net N mineralization rate and pre-incubation exchangeable NO_3^- or pre-incubation exchangeable NH_4^+. The fraction of soil organic N mineralized in 30 days was weakly related to the pre-incubation exchangeable NO_3^- ($p = 0.1$, $r = 0.29$) and NH_4^+ ($p = 0.09$, $r = 0.36$) (Table 3).

Without taking into account the presence of plant residues, the fraction of soil organic N mineralized in 30 days varied among the cropping systems (Table 4). When the samples were not frozen, the continuous corn with winter rye treatment had the highest fraction of soil N mineralized and the unfertilized prairie had the lowest fraction. When the samples were frozen first, the highest fraction of soil N that was mineralized also occurred in samples from the continuous corn with winter rye, and the lowest fraction of soil N that was mineralized was found in samples from the continuous corn treatment.

The relationships between the soil organic C, total N, C/N ratio and the net N mineralization rate indicate that net N mineralization was affected by soil organic matter.

However, our results contrast with those of Ros et al. (2011), who collated net N mineralization data in 98 agricultural soils and reported that soil C, soil N and soil C/N were positively related to net N mineralization. Soil C, N, and C/N might not be reliable laboratory indices of N mineralization. Other researchers recommended that combining indices for predicting mineralizable N is needed and promising (Schomberg et al., 2009; Ros et al., 2011).

2.4.5 Effect of plant residues on N mineralization

Concentration and chemical characteristics of plant residues

The mass of plant residues varied considerably by cropping systems (Table 5). The two prairie systems produced significantly larger amounts of plant residues (1.30 g kg^{-1} in P and 1.41 g kg^{-1} in PF) than the other systems did. However, the analysis of variance (Table 6) demonstrated that the net N mineralization rate (p = 0.61) did not depend on the concentration of plant residues in the soil.

The chemical characteristics of the plant residues that were separated from the soil samples are shown in Table 5. The total C of the plant residues was 288 g kg^{-1} in the CC system, 297 g kg^{-1} in the CCW system, 355 g kg^{-1} in the CS system, 365 g kg^{-1} in the P system, and 377 g kg^{-1} in the PF system. The low C concentration of residue from the corn cropping systems may have reflected an enrichment in biogenic silica as the residues decayed. The total N ranged from 6.7 g kg^{-1} in the P system to 13.3 g kg^{-1} in the CCW system. The C/N ratio among these five cropping systems had a wide range, from 22 in CC and CCW to 55 in P. The analysis of variance (Table 6) explored the effects of the chemical characteristics of the plant residue on net N mineralization rate. There were no

significant effects of plant residues' total C, N, or C/N ratio on net N mineralization rate or on the fraction of organic N mineralized in this experiment. The result contrasts with most authors' conclusions that when the C/N ratio of plant residues is < 25, net N mineralization is likely, and when the C/N ratio is > 30, immobilization occurs (e.g., Trinsoutrot et al. 2000; Kumar and Goh, 2003). Our results suggest that the C/N ratio of the partly decomposed plant residues would not be an effective predictor of N mineralization rates in these cropping systems.

Interactions of plant residues' quantity and quality

The plant residue N per soil weight in Table 5 was calculated by multiplying the total N of plant residues by the residue concentration, so it is a parameter that represents both the quantity and chemical characteristics of the residues. The plant residue N ranged from 6.0 mg N kg^{-1} soil in continuous corn (CC) to 17.0 mg N kg^{-1} soil in N-fertilized prairie (PF). The least significant difference analysis showed that the plant residue N was significantly different in each cropping system (Table 5). The analysis of variance (Table 6) showed that there was a significant effect of plant residue N on net N mineralization rate ($p < 0.001$). The net N mineralization rate was positively related to plant residue N, as shown in Fig. 4 ($n = 20$, $r = 0.65$).

We conclude that in this experiment the rate of N mineralization was not significantly affected simply by the *quantity* or simply by the *chemical characteristics* of the belowground residues of the five cropping systems. But the *interactions* of quantity and quality of the partly decomposed plant residues did affect the N mineralization rate.

2.5 Conclusions

Studies of mineralization of organic N showed that the cumulative amount of mineralized N was linearly related to time of incubation over 30 days. A zero-order kinetics model better represented this short-period incubation study than the first-order kinetics model described by Stanford and Smith (1972).

Freezing and thawing of soil samples increased the net N mineralization rate about twofold. Freezing may change the features of soil organic matter, and make it more easily decomposable. Presumably, N in soil microbial biomass killed by freezing might be a more important source for mineralization. It is possible that, after thawing, the remaining microbial community quickly recovered and released N to the soil inorganic N pool by decomposition of the dead microbial biomass.

Cropping systems had a significant effect on net N mineralization rate. The fertilized perennial prairie system had a higher net N mineralization rate than did corn-based systems. Inclusion of a winter cover crop in a continuous corn system also increased the net N mineralization rate by releasing the N from the cover crop residue.

In this experiment, analysis of variance indicated that soil total N and clay content were related to N mineralization. But due to the narrow range of soil total N and clay content among our samples, the correlation is not clearly expressed between those characteristics and the mineralization rate.

Individually, the presence of plant residues, the residue concentration, and any chemical characteristics did not affect net N mineralization. But by looking at the interactions between the *quantity* and the *chemical characteristics* of the plant residue,

we found that the plant residue N per weight of soil significantly increased N mineralization.

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2.8 Figures

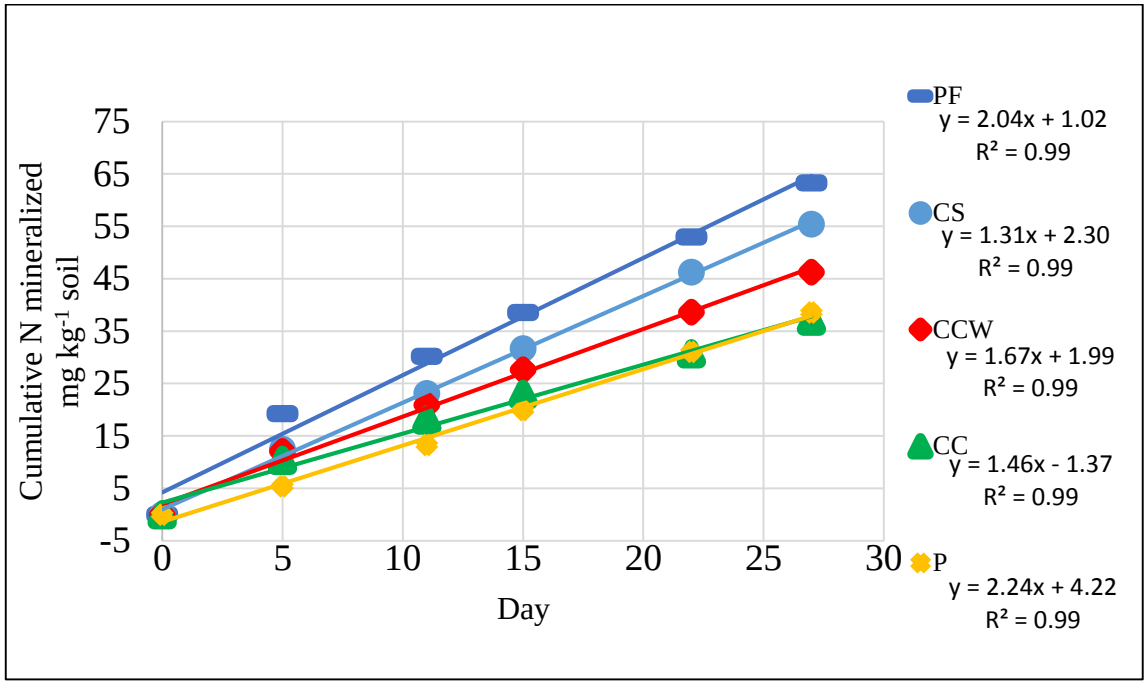

Figure 1. Example of cumulative mineralized N over 30 days for one of the four field replicates of all cropping systems with plant residues intact (WR). The five cropping systems are: continuous corn (CC), continuous corn with rye grown as a winter cover crop (CCW), corn-soybean rotation under corn (CS), reconstructed multispecies prairie (P), and N-fertilized reconstructed multispecies prairie (PF).

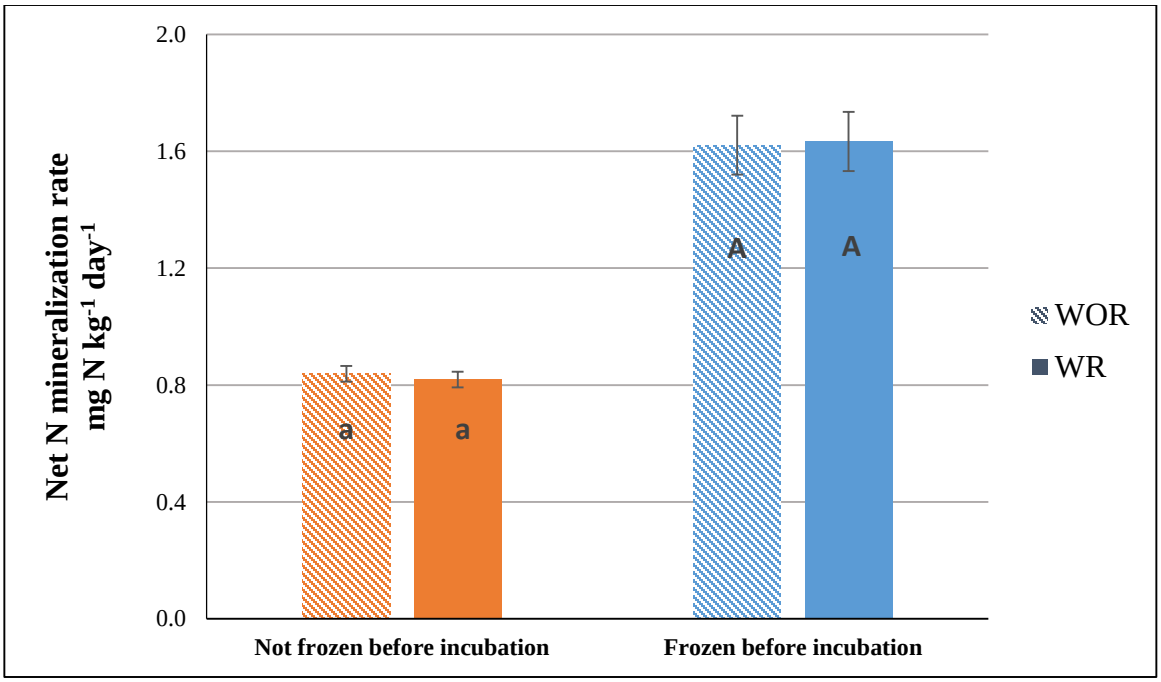

Figure 2. The effect of plant residues on net N mineralization rate under the freezing treatment and under the no-freezing treatment. Lowercase letters indicate the difference between treatments with (WR) and without (WOR) plant residues ($p < 0.05$) under the no-freezing treatment. Uppercase letters indicate the difference between treatments with (WR) and without (WOR) plant residues ($p < 0.05$) under the freezing treatment. Error bars represent standard errors of the mean.

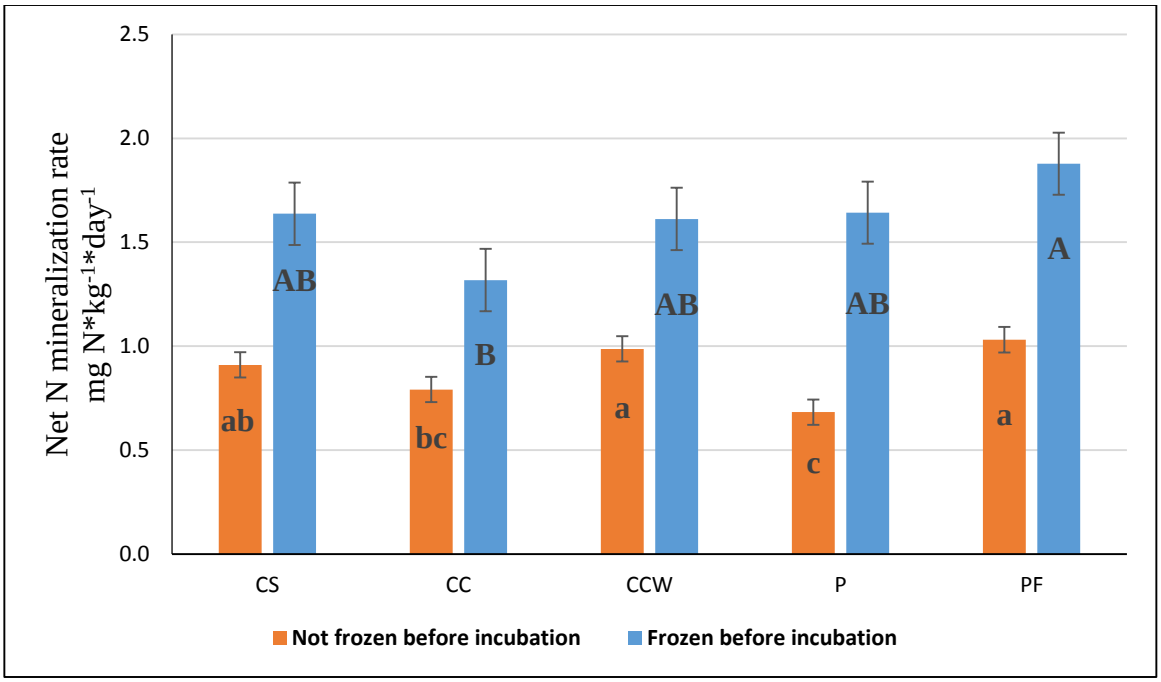

Figure 3. Net N mineralization rate among cropping systems under no-freezing and freezing treatments. Lowercase letters indicate differences among cropping systems ($p < 0.05$) under the no-freezing treatment. Uppercase letters indicate differences among cropping systems ($p < 0.05$) under the freezing treatment. Error bars represent standard errors of the mean. The five cropping systems are: continuous corn (CC), continuous corn with rye grown as a winter cover crop (CCW), corn-soybean rotation under corn (CS), reconstructed multispecies prairie (P), and N-fertilized reconstructed multispecies prairie (PF).

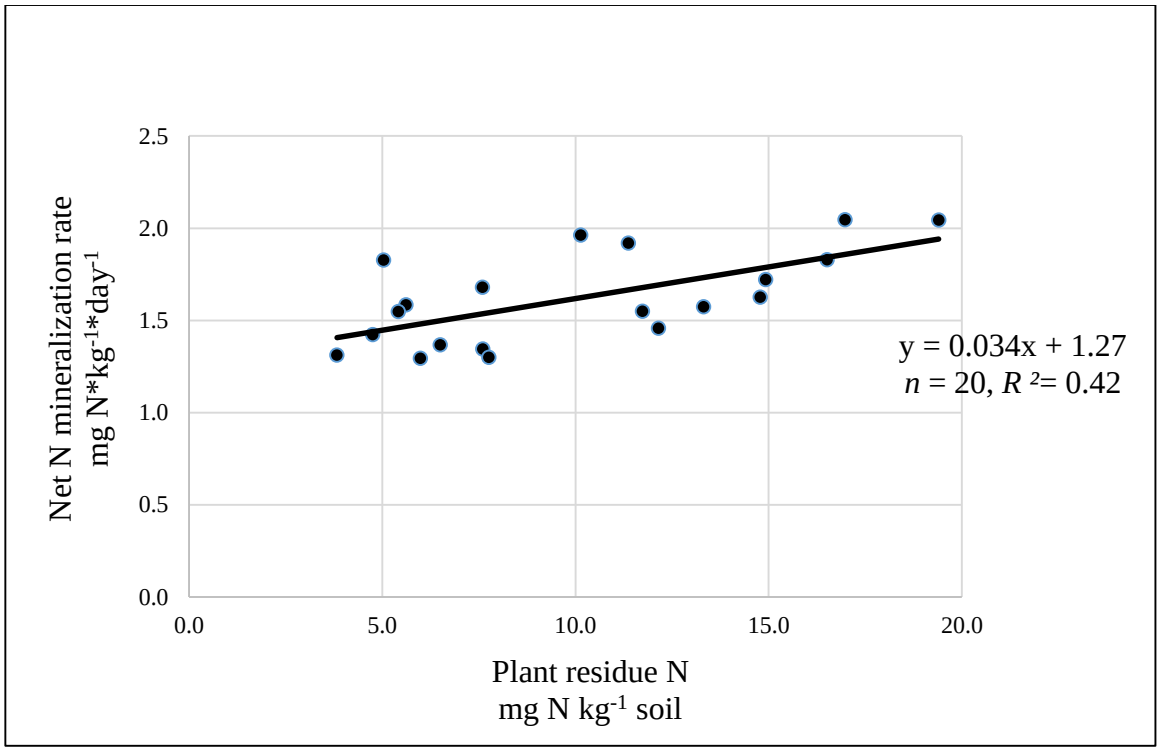

Figure 4. Linear regression of net N mineralization rate of soil samples with plant residues (WR) and under freezing treatment *vs.* plant residue N.

Table 1. Chemical and physical characterististics of soils among the cropping systems.

Cropping systems	**Organic C**†	**Total N**†	**Inorganic N**		**pH**	**Clay**	**Sand**
			NH_4^+	**NO_3^-**	**1:1**		
	---------- g kg^{-1} ----------		-----mg kg^{-1} -----			---- g kg^{-1} ----	
Corn – soybean rotation (CS)	26.2a*	2.2a	0.49a	21.9ab	6.8a	276a	375a
Continuous corn (CC)	25.3a	2.1a	0.50a	28.7a	6.5a	282a	362a
Continuous corn with rye as a winter cover crop	24.1a	2.0a	0.59a	30.8a	6.6a	244a	423a
Reconstructed prairie, unfertilized (P)	25.7a	2.0a	0.77a	0.7c	6.9a	269a	404a
Reconstructed prairie, N-fertilized (PF)	28.8a	2.3a	0.61a	11.8bc	6.7a	282a	373a

† Average of with (WR) and without residue (WOR) samples.
*Means followed by the same letter are not significantly different among cropping systems at $p = 0.05$.

Table 2. Analysis of variance: Effect of cropping systems, freezing treatment and presence of plant residue on net N mineralization rate (n = 80).

Treatments	Effect on Net N mineralization rate *(P < F)*
Cropping systems	0.0038
Freeze or non-freeze	<0.0001
Plant residue presence	0.31

Table 3. Analysis of variance: The effect of soil characteristics on N mineralization in samples without plant residues (WOR) under the no-freeze treatment (n = 30). (NA = not applicable)

Soil characteristics	Net N mineralization rate	Percentage of soil organic N mineralized over 30 days
	----------*P* < *F*----------	
Soil C/N ratio	0.1	NA
Soil total C	0.07	NA
Soil total N	0.04	NA
Clay content	0.005	0.0004
Pre-incubation exchangeable NO_3^-	0.2	0.1
Pre-incubation exchangeable NH_4^+	0.6	0.09

Table 4. Fraction of soil organic N mineralized in 30 days among cropping systems (without regard for the presence of plant residues, $n = 80$).

Cropping system	**Fraction of soil organic N mineralized in 30 days**	
	Not frozen before incubation	**Frozen before incubation**
	----------g N kg^{-1} soil organic N----------	
Corn following soybean	12.4ab*	25.5ab
Continuous corn	11.3ab	22.1b
Continuous corn with rye as a winter cover	13.8a	29.4a
Reconstructed prairie, unfertilized	9.7b	27.2ab
Reconstructed prairie, N-fertilized	12.9ab	26.8ab

*Means within a column that are followed by the same letter are not significantly different among cropping systems at $p = 0.05$.

Table 5. Characteristics of belowground residues isolated from the WOR soil samples (*n* = 40).

Cropping system	Total C	Total N	Mass C:N	Residue concentration	Plant residue N
	g kg^{-1} plant residues			g kg^{-1} soil	mg N kg^{-1} soil
Corn following soybean	355a*	12.4a	28b	0.87b	11.0b
Continuous corn	288b	12.9a	22c	0.46c	6.0e
Continuous corn with winter cover crop (rye)	297b	13.3a	22c	0.58c	7.7d
Reconstructed prairie, unfertilized	365a	6.7b	55a	1.30a	8.8c
Reconstructed prairie, N-fertilized	377a	12.4a	31b	1.41a	17.0a

*Means within a column that are followed by the same letter are not significantly different among cropping systems at $p = 0.05$.

Table 6. Analysis of variance: The effects of plant residue concentration and characteristics on net N mineralization rate in samples with plant residues (WR) under the freezing treatment (n = 30).

Properties of plant residue	Effect on: Net N mineralization rate *(P < F)*
Plant residues concentration	0.61
Plant residues C/N ratio	0.89
Plant residues total C	0.96
Plant residues total N	0.51
Plant residue total N * plant residues concentration	< 0.001

CHAPTER 3. AMINO ACIDS IN SOILS AND CROP RESIDUES

3.1 Introduction

Amino acids are organic molecules that are biologically important for all organisms. All amino acids share a common structure: $RCH(NH_2)COOH$. A central (alpha) carbon bonds with an amine ($-NH_2$) functional group, a carboxylic acid (-COOH) group, a hydrogen atom (-H), and a group of variable composition, symbolized by R, which is called the side chain. The side chain differs with each amino acid. Amino acids can be grouped by polarity, charge, and the type of side chain. Amino acid monomers may be linked by amide bonds to form short chains, which are called peptides. Polymers of peptides are called polypeptides. One or more polypeptides fold and coil into a specific 3-dimensional structure to form a protein. About 20 types of amino acid compose proteins in living organisms, which can also be found in soil. Besides these 20 amino acids, other amino acids which are not normal constituents of proteins are also found in soil. In addition to amino acids, there are two amino sugars that are abundant in soils, i.e., glucosamine and galactosamine (Stevenson, 1982).

Amino acids in soil can exist in three states: free amino acids in the soil solution, amino acids or peptides that are bound to clay particles and soil organic matter, and proteinaceous amino acids in proteins and polypeptides (Schulten and Schnitzer, 1997). Free amino acids can exist in soil solution in macropores or in micropores. Free amino acids are readily decomposed by microorganisms, and they are ephemeral in soil. The

concentrations of free amino acids present in the soil solution represent a balance between synthesis and decomposition by microorganisms. In soil solution and at typical soil pH values, the amine groups of free amino acids are protonated and positively charged ($-NH_3^+$), while the carboxyl groups are deprotonated and negatively charged ($-COO^-$). The charged amino acids can be adsorbed to clay particles and soil organic matter by electrostatic forces. The side chains of basic amino acids such as lysine, arginine, and histidine are also positively charged, and the increased positive charge promotes adsorption of these amino acids to negatively charged clay particles. Amino acids in proteins are bound to one another by peptide bonds and exist as polymers in soil organic matter. The proteinaceous amino acids are the largest fraction of amino acids in soil.

Amino acids are the building blocks of plant proteins. Proteins act as enzymes to catalyze biochemical reactions in cells, or they occur in conjunction with structural units such as the plasma membrane or the plant cell wall. The concentration of amino acids in plants varies greatly depending on the species of the plant, the type of plant tissue, and the growth stage of the plant. Plant samples used in the present experiment were partially decomposed crop residues that were derived from various parts of the crop plants, but especially from roots. There is little available literature about amino acid concentrations in plant roots.

The determination of amino acids in soils usually has three steps: extraction, isolation, and detection. Firstly, the amino acids are extracted from the soil sample; secondly, the individual amino acids are isolated; and at last, the concentration of each amino acid is determined. Amino acids are normally extracted from soil by treatment with hot mineral acids, such as HCl. Martens and Loeffelmann (2003) proposed to extract

soil with methanesulfonic acid (MSA) to avoid the HCl-induced oxidation and degradation of methionine, cysteine, serine, and threonine. Furthermore, MSA is nonvolatile and thermally stable at elevated temperatures (Olk, 2008). The first study to isolate individual amino acids that had been extracted from soil was conducted by Bremner (1950), who isolated amino acids by paper partition chromatography. As chromatographic techniques have developed, amino acid isolation has become easier and more rapid than before. Ion exchange chromatography was applied to amino acid isolation by Stevenson (1954). Gas chromatography has also been used to isolate amino acids in some specific cases (Cheng, 1975). Coupled with the chromatography, the ninhydrin colorimetric auto-analyzer may be used to detect the quantity of each amino acid. Martens and Loeffelmann (2003) were the first to apply pulsed amperometric detection to the analysis of soil amino acids.

In the present experiment, amino acids in soil samples and in plant residue samples were analyzed by using two approaches: high performance liquid chromatography (HPLC), which couples cation exchange chromatography with a ninhydrin colorimetric method, and high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The objective of this experiment was to compare the precision and effectiveness of the HPLC-ninhydrin colorimetric method and the HPAEC-PAD method, as well as to document the concentration of amino acids in partially decomposed plant residues.

3.2 Methods and materials

3.2.1 Sample collection and preparation

Soil samples and plant residue samples used in this study were the same samples as we used in the mineralization study described in Chapter 2. They were collected in June 2013 from the five cropping systems at the Comparison of Biofuel Systems (COBS) site. Soil samples were air-dried and then finely ground and passed through a 0.1-mm sieve. Plant residues that were separated from soil samples were washed with distilled water to remove adhering soil materials before they were oven-dried at 70 ℃ for 3 d. Then the plant residue samples were finely ground and passed through a 0.1-mm sieve.

3.2.2. Extraction

The same extraction procedure was used in both the HPAEC-PAD (hereafter, amperometric) method and the HPLC-ninhydrin colorimetric (hereafter, ninhydrin) method. Two mL of 4 M methanesulfonic acid (MSA) containing 0.2% (w/w) tryptamine were added to 250 mg of finely ground air-dried soil or 20 mg of finely ground plant residue sample (oven-dried at 700C for 3 days). Tryptamine was added to prevent the decomposition of acid-sensitive amino acids and to speed up the hydrolysis of amino compounds (Simpson et al., 1976). The sample-acid mixture was autoclaved at 121 ℃ for 16 h. When cooled, the pH of the digest was first adjusted to 2.1 to 2.5 with NaOH and diluted to 10-mL with deionized water; then an aliquot was taken for the ninhydrin method. For the amperometric method, another 2-mL aliquot was taken from the 10-mL extract and its pH was further adjusted to 4 to 6. Then the volume of the 2-mL aliquots was adjusted to 10 mL with deionized water. Digests were centrifuged and filtered

through a 0.2-µm 25-mm nylon syringe filter (Tisch Scientific Inc., OH) after adjusting of pH and volume. The filtrates were kept frozen before analysis. Before analysis, each diluted digest was thawed at room temperature.

3.2.3 HPAEC-PAD (amperometric method)

A subaliquot of each soil and plant residue extract was first passed through an anion exchange chromatographic column to separate the amino acids for pulsed amperometric detection. Amino acids that arrived at the amperometric detector were oxidized on a Au surface. The electrical flow produced by the oxidation of each amino acid was precisely measured by the detector (Olk, 2008). The amino acids that can be detected and quantified by the amperometric method are: arginine, ornithine, lysine, alanine, threonine, glycine, valine, hydroxyproline, serine, proline, isolecucine, lecucine, methionine, histidine, phenylalanine, glutamic acid, aspartic acid, cysteine (a cysteine dimer), tyrosine; and the two amino sugars galactosamine and glucosamine.

3.2.4 HPLC and ninhydrin colorimetric method

A second subaliquot of each soil and plant residue extracts was maintained at pH 2.1 to 2.5 so that the amino acids remain protonated. These cationic forms were first chromatographically separated on a cation exchange column by high performance liquid chromatography. Then the amino acids were derivatized (post-column) with ninhydrin (triketohydrindene hydrate), which reacts with the α-amino N of amino acids in acidic solution to form a purple product: diketohydrindylidenediketo-hydrindamine (DYDA). The concentration of DYDA was determined by light absorption at 440 nm for proline,

and at 570 nm for all other amino acids (Yemm et al., 1955; Olk, 2008). The amino acids that the ninhydrin method can detect are: arginine, lysine, alanine, threonine, glycine, valine, serine, proline, isolecucine, lecucine, methionine, histidine, phenylalanine, glutamic acid, aspartic acid, cystine, and tyrosine.

3.3 Results and discussion

3.3.1 Characteristics of soil samples and plant residue samples

The characteristics of the soil samples employed in this study are described in the previous chapter of this thesis (Chapter 2, Table 1). There was slightly more organic C and total N in soil from the fertilized prairie system than in soil from other cropping systems, but the differences were not statistically significant. Chemical characteristics of the plant residues are also given in Chapter 2 of the thesis, Table 3. The total C of the plant residues in the continuous corn and the corn with winter rye systems was significantly lower than total C in the corn-soybean rotation, prairie, and fertilized prairie systems. The total N of the plant residues in the prairie cropping system was significantly lower than the total N in the other cropping systems. The C/N ratio among these five cropping systems had a wide range, from 22 to 55. The prairie system had the highest C/N ratio of all five cropping systems.

3.3.2 Comparison of the amperometric method with the ninhydrin method

The concentrations of amino acids extracted from the soil samples were measured by the amperometric method (19 amino acids and 2 amino sugars) and the ninhydrin method (17 amino acids). Ornithine, hydroxyproline and amino sugars can be detected

only by the amperometric method. The measured concentrations of ornithine and hydroxyproline were negligible compared to the total concentration of amino acids. So when comparing the amperometric and ninhydrin methods, the concentrations of amino sugars, ornithine, and hydroxyproline, were not included. The concentrations of amino acids were converted to amino acid-N (AA-N) for comparison purposes. Concentrations of each amino acid in all samples are attached in Table 3 and Table 4 of the appendix.

In the soil samples collected under different cropping systems, the average total amino acid-N ranged from 604 to 662 mg kg^{-1} soil measured by the amperometric method and from 604 to 717 mg kg^{-1} soil measured by the ninhydrin method (Table 1). The average ninhydrin measurements of total amino acid-N were variable but generally similar to the amperometric measurements (Table 1). When plotted on an individual basis (before averaging by cropping systems treatment), the amperometric total amino acid-N concentrations were linearly related to those of the ninhydrin method (Figure 1). The amperometric method includes an anion exchange chromatography step, and it recovered more basic amino acids than the ninhydrin method, which employs cation exchange chromatography for amino acid separation. While the ninhydrin method recovered more acidic amino acids than the amperometric method.

As presented in Table 2, the concentration of basic amino acids (i.e., the sum of lysine-N, arginine-N, and histidine-N) measured by the amperometric method was nearly twice that measured by the ninhydrin method. The determination of acidic amino acids was just the opposite. The sum of aspartic acid-N and glutamic acid-N measured by the ninhydrin method was 4-6 times more than that measured by the amperometric method. When the concentrations of N of amino acids with ionizable side chains (lysine, arginine,

histidine, aspartic acid, and glutamic acid) were subtracted from the total amino acid-N, the N concentrations of the remaining amino acids measured by the two methods were more closely related to each other (R^2 = 0.79, Fig. 1) than before subtraction (R^2 = 0.65, Fig. 1). However, preferential separation and recovery by chromatography did not explain why the soil total amino acid-N in the ninhydrin measurement was greater than that in amperometric measurement, because the magnitude of the difference in acidic and basic amino acids was about the same. Further research is needed to explain the differences in the determination of soil amino acids by using the amperometric method and ninhydrin method.

The range of amino acid-N concentrations in the plant residue samples was wider than that of amino acid-N in soil samples. The total amino acid-N measured by the amperometric method exceeded the concentration of total N in some plant samples (Table 2). As indicated in Fig. 2, the total amino acid-N values of the plant residue samples measured by the amperometric method were not linearly related to the ninhydrin measurements (y = -0.026x + 6815, n = 20, R^2= 0.002). When the concentrations of individual amino acids measured by the two methods were compared, we found that arginine concentrations measured by the amperometric method were about 4 times greater than those measured by the ninhydrin method. The abnormally high arginine peak obtained in the amperometric method may have reflected other compounds in the plant residue samples that co-eluted with the amino acids. When the concentration of arginine N was subtracted from the total amino acid-N of both methods, the values of amino acid-N measured by the two methods were still not linearly related to each other (y = 0.013x + 4112, n = 20, R^2= 0.001) (Fig. 2). But after dropping arginine, the amperometric

measurement was then in the same range as the ninhydrin measurement (Fig. 2). This abnormal result for arginine in plant samples has also been reported in other laboratories (Olk, 2015, personal communication). It is likely that some carbohydrate molecules, such as glucose, which is abundant in plant residues, might be extracted and eluted through the chromatographic column with the first amino acids, overlapping with the arginine peak (and perhaps with the alanine and threonine peaks). Insertion of a commercially available carbohydrate trap before the chromatography step in the analysis could improve the determination of arginine, but first mass spectrometry should be used to confirm the identity of any co-eluting compounds. We recommend that the amperometric method to measure amino acids in plant samples be avoided at this time; the ninhydrin method appears to be the better choice for plant samples.

3.3.3 The concentration of amino acid-N

Soil

In addition to the concentration of total amino N, Table 1 presents the fraction of amino N in soil organic N. Using high performance ion-exchange chromatography coupled with the ninhydrin colorimetric method, Senwo and Tabatabai (1998) reported that the total amino acids in cultivated Iowa soils typically ranged from 566 to 1022 mg kg^{-1} soil. Compared to Senwo and Tabatabai's results, our results are at the lower boundary of their range. Considered as a fraction of soil organic N, the amino acid-N ranged from 262 to 275 g kg^{-1} soil organic N (26-28%) as measured by the amperometric method and from 260 to 287 g kg^{-1} soil organic N (26-29%) as measured by the ninhydrin method. For soils associated with Iowa corn-soybean cropping systems,

Martens et al. (2006) reported that an average of 51% of total N was amino acid-N, with a range from 35 to 76%, as measured by the amperometric method. Our results were lower than those of Martens et al. (2006). Neither the absolute concentration of soil amino acid-N, nor the fraction of amino acid-N in soil organic N (measured by either of the methods), varied significantly among the soils of the five cropping systems.

Plant residues

As discussed in the method comparison section, the ninhydrin method was more reliable than the amperometric method for measurement of amino acid-N in plant materials. Therefore we discuss only the amino acid-N measured by the ninhydrin method (Table 3). The total amino acid-N did not vary among the residues of the cropping systems, except under the prairie system (2.6 g kg^{-1} residue), which was half the amount of other cropping systems (ranged from 5.2 to 5.7 g kg^{-1} residue) (Table 3). But the concentration of total N in residues of the unfertilized prairie system (6.7 g N kg^{-1} residue) was also about half that of the other cropping systems (about 12.5 g N kg^{-1} residue) (Chapter 2, Table 3). The fraction of amino acid-N in plant residues' total N did not vary significantly among residues of the cropping systems, ranging from 389 to 457 g kg^{-1} plant residues total N (39-46%). The dry matter of green plant tissues typically are ~7% crude protein (calculated by assuming that all proteins are about 16% N) (Bosworth, 2005), which is 70 g protein kg^{-1} plant dry matter. In the present study, the average concentration of total amino acids in plant residues from the five cropping systems was 36 g amino acids kg^{-1} plant residues. Compared to the freshly dried plant samples, the total amino acid concentration of the dried and partially decomposed plant residues

(mainly roots) was lower. There is little available literature about amino acid concentrations in plant roots.

3.4 Conclusions

The concentration of amino acids in all the soil samples were similar, as measured by the amperometric method and the ninhydrin method. The anion exchange chromatography (HPAEC) method was better to measure basic amino acids, whereas the cation exchange chromatography (HPLC-ninhydrin) method was better to measure acidic amino acids. These two method could be combined to recover the maximum amount of amino acids in future studies.

On the other hand, in plant samples, the ninhydrin method could be a better choice than the amperometric method at present, perhaps because of the peaks of some amino acids were overlapped by co-eluting carbohydrate peaks. However, we think the amperometric method could be improved by insertion of a commercially available carbohydrate trap before the chromatography step, after identifying the carbohydrates molecules.

The total amino acid-N of plant residue samples measured by the ninhydrin method was 2.6 g kg^{-1} under the prairie system, and about 5.5 g kg^{-1} under continuous corn, continuous corn with winter rye, corn-soybean rotation, and N-fertilized prairie systems. About forty percent of total N in the partially decomposed plant residues was amino acid-N. Compared to the freshly dried plant samples, the total amino acid concentration of the dried and partially decomposed plant residues (mainly roots) was lower.

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3.6 Figures

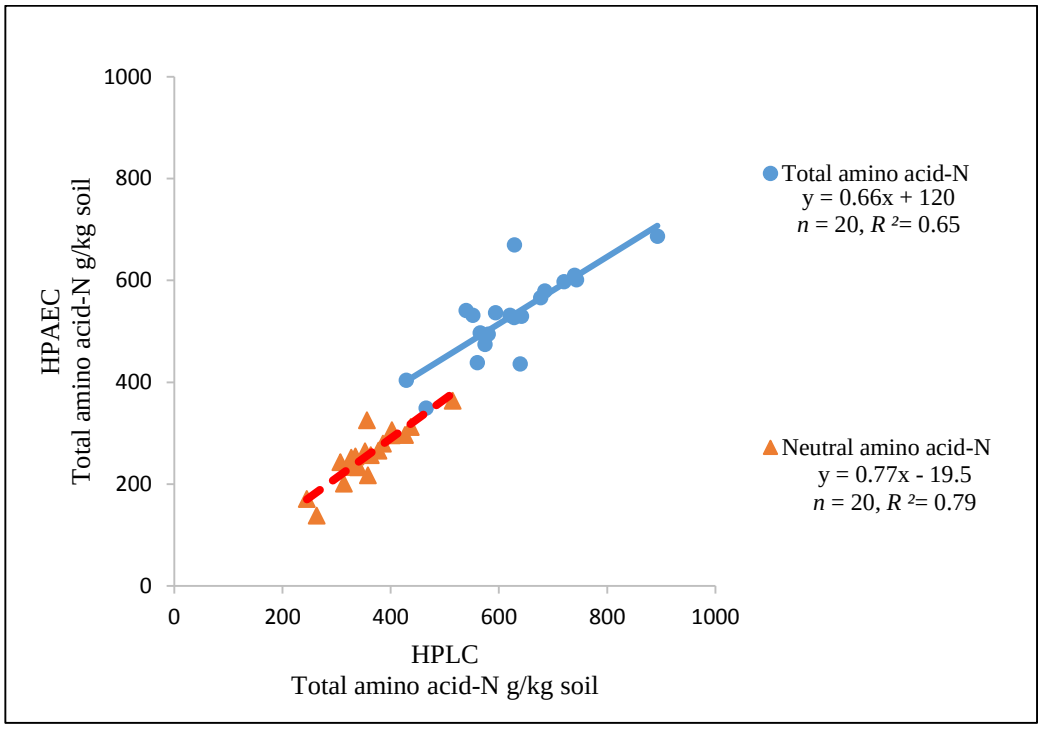

Figure 1. Comparison of total amino acid-N and neutral amino acid-N in soil samples of all the research plots (without regard for cropping system) between the amperometric method (HPAEC) and the ninhydrin method (HPLC).

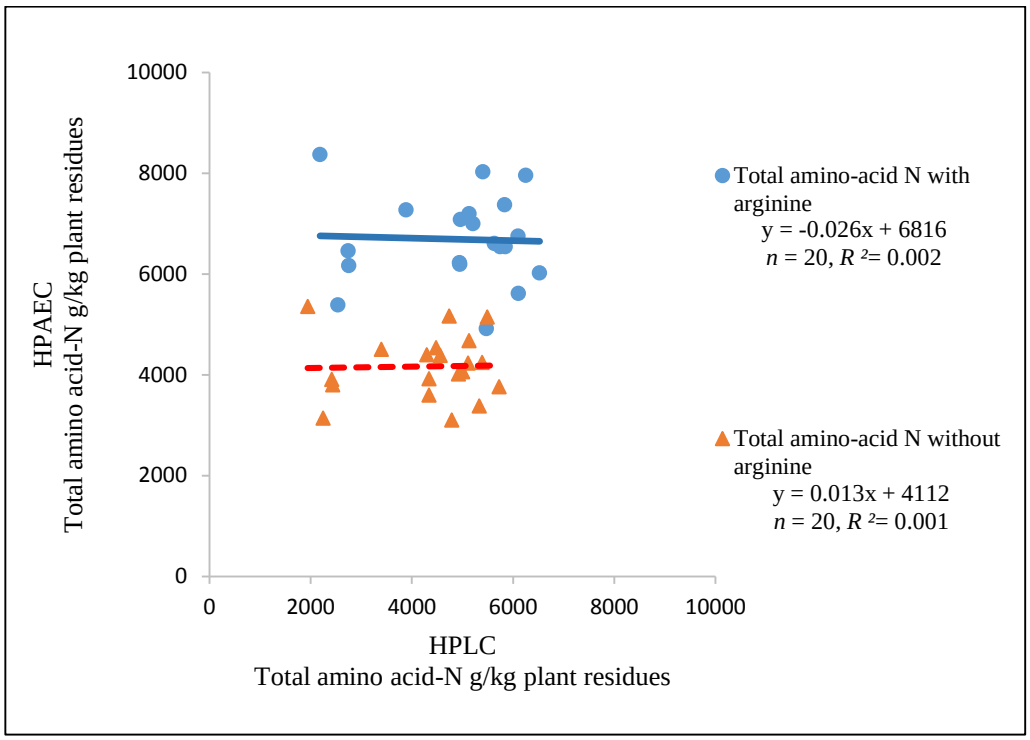

Figure 2. Comparison of total amino acid-N in plant residue samples from all the research plots (without regard for cropping system) between the amperometric method (HPAEC) and the ninhydrin method (HPLC).

3.7 Tables

Table 1. Amino acids in soils associated with each cropping system measured by the amperometric method (HPAEC) and the ninhydrin method (HPLC). Values reported in the amperometric method include the two amino sugars: galactosamine and glucosamine.

Cropping systems	**HPLC**	**HPAEC**	**HPLC**	**HPAEC**
	----------mg AA-N / kg soil---------		---g AA-N / kg soil organic N---	
Corn following soybean	604a*	614a	266a	273a
Continuous corn	605a	614a	271a	275a
Continuous corn with winter cover crop (rye)	605a	607a	263a	265a
Reconstructed prairie, unfertilized	589a	604a	260a	262a
Reconstructed prairie, N-fertilized	717a	662a	287a	268a

*** Means followed by the same letter are not significantly different at $p = 0.05$.**

Table 2. The difference between the amperometric and ninhydrin methods for acidic or basic amino acid-N measured by the amperometic method (HPAEC) and the ninhydrin method (HPLC) in soil samples associated with each cropping system.

Cropping systems	**Acidic amino acids** Sum of glutamic acid-N, and aspartic acid-N			**Basic amino acids** Sum of arginine-N, lysine-N and histidine-N		
	HPAEC	**HPLC**	**Difference**	**HPAEC**	**HPLC**	**Difference**
	----------AA-N mg / kg soil----------					
Corn following soybean	30	140	**110**	241	117	**124**
Continuous corn	28	136	**109**	240	118	**122**
Continuous corn with winter cover crop (rye)	28	141	**113**	241	115	**126**
Reconstructed prairie, unfertilized	30	138	**108**	241	113	**128**
Reconstructed prairie, N-fertilized	31	173	**142**	254	136	**118**

Table 3. Amino acids in plant residues associated with each cropping system measured by the amperometric method (HPAEC) and the ninhydrin method (HPLC). Values reported in the amperometric method include the two amino sugars: galactosamine and glucosamine.

Cropping systems	**HPLC**	**HPAEC**	**HPLC**	**HPAEC**
	-------g AA-N / kg residue-----		----g AA-N / kg residue total N----	
Corn following soybean	5.1a*	7.9a	414a	635b
Continuous corn	5.7a	7.6a	437a	587b
Continuous corn with winter cover crop (rye)	5.7a	7.6a	429a	570b
Reconstructed prairie, unfertilized	2.6b	7.6a	389a	1140a
Reconstructed prairie, N-fertilized	5.5a	8.2a	457a	682b

*** Means followed by the same letter are not significantly different at *p* = 0.05.**

CHAPTER 4. GENERAL CONCLUSIONS

4.1 Nitrogen mineralization study

The first objective of this study was to determine and compare net N mineralization rates in five Midwest cropping systems. Overall, cropping systems had a significant effect on net N mineralization rate. The fertilized perennial prairie systems had a higher net N mineralization rate than corn-based systems. Winter cover crop included in continuous corn also increased the net N mineralization rate by releasing the N from cover crop residue.

The second objective was to evaluate the effects of the quantity and the chemical characteristics of the decomposing plant residues of each cropping system on N mineralization. Individually, the presence of plant residues, the residue concentration, and the chemical characteristics of the residues did not affect net N mineralization. However, by looking at the *interactions* between the quantity and the chemical characteristics of the plant residue, we found the plant residue N per weight of soil was significantly correlated with increased N mineralization.

The third objective was to determine if any soil characteristics affected N mineralization in the selected cropping systems. In this experiment, analysis of variance indicated that the net N mineralization rate was strongly affected by clay content, moderately affected by soil total N, and weakly affected by soil C/N ratio and soil organic C. But due to the narrow ranges of soil total N, soil organic C, soil C/N ratio, and clay content among our samples, the correlation is not clearly expressed between those characteristics and the mineralization rate.

The fourth objective was to evaluate the freezing-thawing effect on N mineralization. Freezing and thawing of soil samples increased the net N mineralization rate about twofold.

4.2 Amino acid study

The first objective of the amino acids study was to compare the precision and effectiveness of the HPLC-ninhydrin colorimetric method and the HPAEC-PAD method, when measuring amino acids in soil samples and plant residue samples. The concentrations of amino acids in soil samples were similar as measured by the amperometric method and the ninhydrin method. The anion exchange chromatography (HPAEC) method was better to measure basic amino acids, whereas the cation exchange chromatography (HPLC-ninhydrin) method was better to measure acidic amino acids. These two method could be combined to recover the maximum amount of amino acids in future studies.The concentrations of amino acids in plant residues samples measured by the amperometric method were much higher than those measured by the ninhydrin method, due to the co-elution of carbohydrates in the amperometric method. We recommend using the ninhydrin method to measure amino acids in plant samples at present. However, we think the amperometric method could be improved by insertion of a commercially available carbohydrate trap before the chromatography step, after identifying the carbohydrates molecules.

The second objective was to document the concentration of amino acids in partially decomposed plant residues. The total amino acid-N of plant residue samples measured by the ninhydrin method was 2.6 g kg^{-1} under the prairie system, and about 5.5

g kg^{-1} under continuous corn, continuous corn with winter rye, corn-soybean rotation, and N-fertilized prairie systems. About forty percent of total N was amino acid-N in the partially decomposed plant residues. Compared to the freshly dried plant samples, the total amino acid concentration of the dried and partially decomposed plant residues (mainly roots) was lower.

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APPENDIX. ADDITIONAL FIGURES AND TABLES

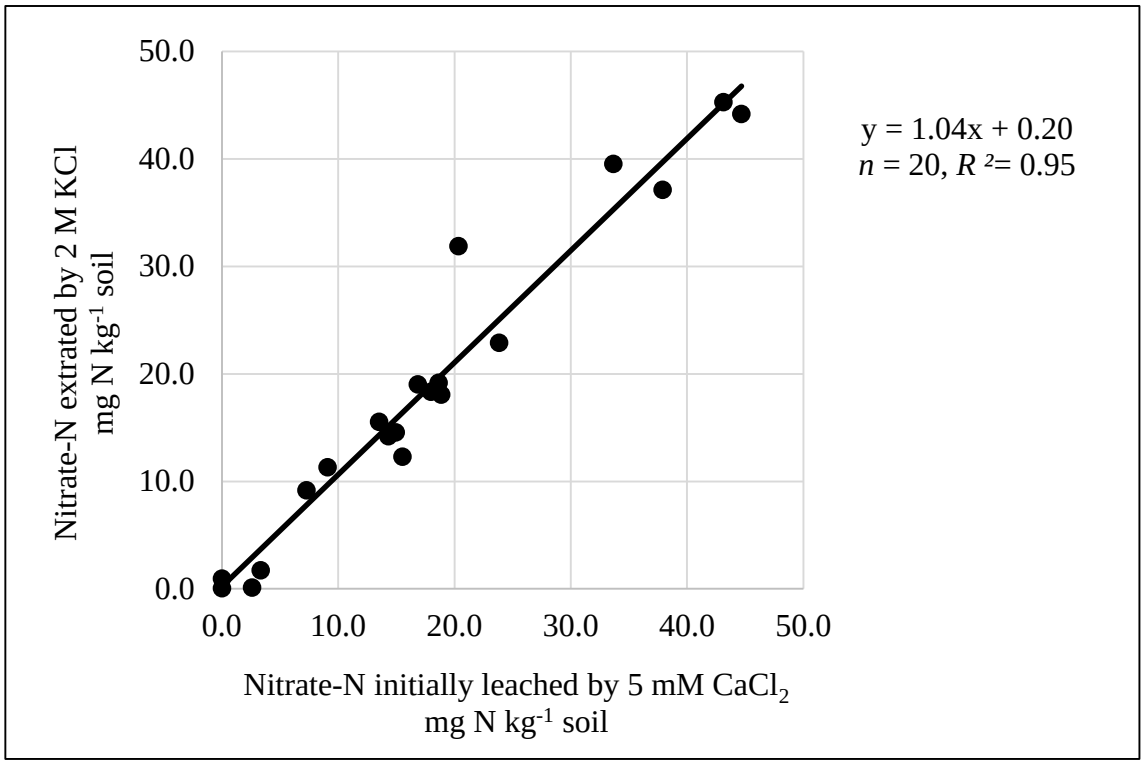

Figure 1. Regression of the nitrate-N obtained in 5 mM $CaCl_2$ initial leaching *vs.* in 2 M KCl extraction before incubation.

Table 1. Chemical and physical characterististics of soils among the cropping systems.

Cropping system	Total N†	Organic C†	C:N†	Extractable NO_3^-	Extractable NH_4^+	pH H_2O	Moisture	Sand	Clay
	---------g / kg soil-------			---------mg N / kg soil-------			----------g / kg soil----------		
Corn following soybean	2.2*±0.1#	26.2±1.2	12.2±0.2	21.88±3.35	0.49±0.05	6.8±0.2	213±11	375±69	276±47
Continuous corn	2.1±0.1	25.3±1.4	12.2±0.3	28.74±7.09	0.50±0.04	6.5±0.2	196±14	362±50	282±31
Continuous corn with winter cover crop (rye)	2.0±0.2	24.1±1.7	12.0±0.3	30.82±6.96	0.59±0.09	6.6±0.2	215±10	423±63	244±46
Reconstructed prairie, unfertilized	2.0±0.2	25.7±2.1	12.6±0.4	0.73±0.39	0.77±0.15	6.9±0.2	225±10	404±95	269±61
Reconstructed prairie, N-fertilized	2.3±0.4	28.8±1.6	12.5±0.2	11.76±1.05	0.61±0.07	6.7±0.2	223±15	373±59	282±31

† **average measured in both with (WR) and without residue (WOR) samples.**
***average of four field replicates.**
#: **standard error of four field replicates.**

Table 2. Characteristics of belowground residues isolated from the soil samples associated with five cropping systems.

Cropping systems	**Total C**	**Total N**	**C:N**	**Plant residue concentration** g plant residues / kg soil
	---------g / kg residue-------			
Corn following soybean	355* ±15[#]	12.4 ±0.4	28.5 ±1.1	0.87 ±0.14
Continuous corn	288 ±7	12.9 ±0.3	22.3 ±0.9	0.46 ±0.06
Continuous corn with winter cover crop (rye)	297 ±7	13.3 ±0.2	22.4 ±1.0	0.58 ±0.15
Reconstructed prairie, unfertilized	365 ±13	6.7 ±0.4	54.7 ±1.2	1.30 ±0.15
Reconstructed prairie, N-fertilized	377 ±8	12.4 ±1.3	31.2 ±2.6	1.41 ±0.16

***average of four field replicates.**
[#]standard error of four field replicates.

Table 3. Concentration of each amino acid in soil samples, measured by high performance anion exchange chromatography coupled with the pulsed amperometric detection (HPAEC) method (A as abbreviation), and high performance liquid chromatography coupled with the ninhydrin colorimetric method (HPLC) (L as abbreviation).

Cropping systems	**Corn-soybean rotation**		**Continuous corn**		**Corn with winter rye**		**Prairie**		**N-fertilized prairie**	
Methods	A	L	A	L	A	L	A	L	A	L
Amino acids	----------amino acid-N mg / kg soil----------									
Arginine	179	59	178	58	181	59	181	58	190	70
Lysine	63	29	62	32	59	28	60	28	64	34
Histidine	0	29	0	28	0	28	0	27	0	32
Aspartic acid	20	90	19	86	19	91	20	89	21	112
Glutamic acid	10	50	9	50	9	50	10	50	10	61
Serine	33	37	32	41	32	40	31	37	36	47
Threonine	28	32	28	34	28	34	28	33	32	40
Cystine	2	2	2	2	2	2	2	2	2	2
Tyrosine	5	9	5	8	5	8	5	9	6	9
Ornithine	9	-	7	-	6	-	8	-	7	-
Glycine	58	80	58	82	57	81	55	77	61	94
Alanine	43	57	43	57	42	57	41	55	47	67
Valine	24	29	24	29	24	29	25	28	27	35
Leucine	17	34	17	34	17	34	18	33	21	39
Isoleucine	13	31	13	32	13	31	13	30	15	35
Methionine	1	0	1	0	2	0	1	0	3	0
Phenylalanine	0	15	0	15	0	15	0	15	0	17
Proline	19	19	19	18	19	19	19	19	22	23
Hydroxyproline	4	-	3	-	4	-	4	-	4	-

Table 3 Continued.

Galactosamine	28	-	29	-	27	-	26	-	29	-
Glucosamine	59	-	67	-	60	-	58	-	65	-

Table 4. Concentration of each amino acid in plant residue samples, measured by high performance anion exchange chromatography coupled with the pulsed amperometric detection (HPAEC) method (A as abbreviation), and high performance liquid chromatography coupled with the ninhydrin colorimetric method (HPLC) (L as abbreviations).

Cropping systems	**Corn-soybean rotation**		**Continuous corn**		**Corn with winter rye**		**Prairie**		**N-fertilized prairie**	
Methods	A	L	A	L	A	L	A	L	A	L
Amino acids	----------amino acid-N mg / kg plant residues----------									
Arginine	2348	648	2278	702	2338	689	2380	293	2425	689
Lysine	801	252	772	233	782	244	802	182	840	233
Histidine	0	226	0	243	0	243	0	145	0	238
Aspartic acid	201	498	185	556	187	555	194	240	207	513
Glutamic acid	94	426	85	457	88	457	92	217	99	456
Serine	387	335	365	369	360	378	361	179	416	353
Threonine	356	271	332	314	328	304	337	139	374	290
Cystine	25	24	24	28	24	30	24	11	27	25
Tyrosine	58	271	50	314	54	304	58	139	63	290
Ornithine	127	-	119	-	127	-	132	-	134	-
Glycine	788	487	755	548	743	558	730	237	793	537
Alanine	562	423	534	465	524	468	525	221	583	463
Valine	376	270	359	287	335	299	341	134	406	293
Leucine	227	389	206	422	201	429	204	178	236	418
Isoleucine	162	269	149	295	145	295	147	116	171	284
Methionine	25	86	21	140	18	145	21	6	26	127
Phenylalanine	0	154	0	169	0	172	0	77	0	167
Proline	228	264	117	288	209	300	216	123	250	288
Hydroxyproline	46	-	34	-	33	-	34	-	47	-

Table 4 Continued.

Galactosamine	324	-	326	-	305	-	300	-	326	-
Glucosamine	754	-	813	-	745	-	743	-	783	-